

Observational Study

Liver fat deposition and mitochondrial dysfunction in morbid obesity: An approach combining metabolomics with liver imaging and histology

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Abstract

AIM: To explore the usefulness of magnetic resonance imaging (MRI) and spectroscopy (MRS) for assessment of non-alcoholic fat liver disease (NAFLD) as compared with liver histological and metabolomics findings.

METHODS: Patients undergoing bariatric surgery following procedures involved in laparoscopic sleeve gastrectomy were recruited as a model of obesity-induced NAFLD in an observational, prospective, single-site, cross-sectional study with a pre-set duration of 1 year. Relevant data were obtained prospectively and surrogates for inflammation, oxidative stress and lipid and glucose metabolism were obtained through standard laboratory measurements. To provide reliable data from MRI and MRS, novel procedures were designed to limit sampling variability and other sources of error using a 1.5T Signa HDx scanner and protocols acquired from the 3D or 2D Fat SAT FIESTA prescription manager. We used our previously described ^1H NMR-based metabolomics assays. Data were obtained immediately before surgery and after a 12-mo period including histology of the liver and measurement of metabolites. Values from ^1H NMR spectra obtained after surgery were omitted due to technical limitations.

RESULTS: MRI data showed excellent correlation with the concentration of liver triglycerides, other hepatic lipid components and the histological assessment, which excluded the presence of non-alcoholic steatohepatitis (NASH). MRI was sufficient to follow up NAFLD in obese patients undergoing bariatric surgery and data suggest usefulness in other clinical situations. The information provided by MRS replicated that obtained by MRI using the $-\text{CH}_3$ peak (0.9 ppm), the $-\text{CH}_2-$ peak (1.3 ppm, mostly triglyceride) and the $-\text{CH}=\text{CH}-$ peak (2.2 ppm). No patient depicted NASH. After surgery all patients significantly decreased their body weight and steatosis was virtually absent even in patients with previous severe disease. Improvement was also observed in the serum concentrations of selected variables. The most relevant findings using metabolomics indicate increased levels of triglyceride and monounsaturated fatty acids in severe steatosis but those results were accompanied by a significant depletion of diglycerides, polyunsaturated fatty acids, glucose-6-phosphate and the ATP/AMP ratio. Combined data indicated the coordinated action on mitochondrial fat oxidation and glucose transport activity and may support the consideration of NAFLD as a likely mitochondrial disease. This concept may help

to explain the dissociation between excess lipid storage in adipose tissue and NAFLD and may direct the search for plasma biomarkers and novel therapeutic strategies. A limitation of our study is that data were obtained in a relatively low number of patients.

CONCLUSION: MRI is sufficient to stage NAFLD in obese patients and to assess the improvement after bariatric surgery. Other data were superfluous for this purpose.

Key words: Fatty liver disease; Magnetic resonance imaging; Lipids; Magnetic resonance spectroscopy; Metabolomics; Mitochondrial function; Morbid obesity; Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis

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Core tip: Despite remaining obese, non-alcoholic fatty liver disease (NAFLD) regressed in patients undergoing bariatric surgery. Magnetic resonance imaging was sufficient to assess these findings and spectroscopy and measurement of liver fat content were superfluous for this purpose. Patients were free of hepatocyte ballooning or fibrosis and results may limit further the indication for liver biopsy to patients with known factors affecting the progression of liver disease. Additional information on the dissociation between lipid storage in adipose tissue and NAFLD suggests the presence of mitochondrial and inflammatory disease and may focus on the unmet need for plasma biomarkers of circulating metabolites produced during altered cellular fatty oxidation and glycolysis.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is an important cardiovascular and metabolic risk factor defined as a higher than 5% intra-cytoplasmic fat deposition in the liver in the absence of alcohol consumption, toxin exposure or viral disease^[1,2]. Because a progressive course is possible in this condition, a benign prognosis should not be assumed. This is particularly disturbing because the prevalence of NAFLD is constantly increasing (now > 30% in adults

in developed countries). This has been attributed to the increased lifespan and the upsurge in obesity but these associations are not clinically well substantiated. For example, the presence of moderate NAFLD is common in young patients with morbid obesity^[3,4]. The effect of weight loss on NAFLD is beneficial in most patients but difficult to achieve with simple advice on changes in lifestyle. In patients with morbid obesity, bariatric surgery is an alternative and effective treatment to obtain a rapid and sustained weight loss. Additionally, other beneficial effects for associated metabolic conditions, apparently unrelated to the decrease in body weight, have been described^[5]. Accurate mechanisms of action, however, remain poorly understood.

There are no reliable non-invasive markers to assess the beneficial effects of surgical procedures on NAFLD and other obesity-associated morbidities. The sensitivity and accuracy of ultrasound are too low to detect NAFLD in obese patients and liver biopsy is the only method to assess the presence of non-alcoholic steatohepatitis (NASH) and/or fibrosis, which characterize the progression in NAFLD. Therefore, the early diagnosis of subclinical NAFLD is uncommon in clinical practice because physicians and/or patients usually find liver biopsy to be a difficult undertaking, which requires specific training so as to ensure the lowest rate of complications^[6,7]. The consequence is that NAFLD, a condition that may lead to eventual liver-related mortality, is frequently understated^[8].

Determination of the hepatic triglyceride concentration^[9] may be useful but we have based our hypothesis on previous findings in animal models^[10] indicating that cholesterol and fatty acids in the liver are better indicators of inflammation, and consequently ¹H NMR metabolic profiling of multiple lipid metabolites in liver extracts may extend our understanding of underlying mechanisms. We also hypothesized that this approach could add useful information to magnetic resonance imaging (MRI) and spectroscopy (MRS) findings in order to explore hepatic lipid mobilization. Finally, we reasoned that correction of possible confounding factors may improve the clinical application of MRI in the assessment of NAFLD in obese patients and its follow-up after bariatric surgery.

MATERIALS AND METHODS

Study population

This is an observational, prospective, single-site, cross-sectional study with a pre-set duration of 1 year involving the recruitment of 47 patients. Only 19 patients were finally included. We discarded 3 patients due to the confirmation of clandestine high consumption of alcohol or prescribed medication that could alter liver function. Other patients refused either the MRI procedure or the biopsy after surgery ($n = 8$). Other patients ($n = 9$) simply did not fit into the apparatus. Finally, the obtained spectra were

considered suboptimal in 8 patients due to excessive proximity between the coil and the gantry. Subsequent studies indicate that to obtain 100% of valid spectra, the width at the level of iliac crests should be less than 100 cm. The local ethics committee, which acts as the institutional review board of the Hospital de Sant Joan de Reus, approved the study protocol and procedures, and written informed consent was obtained from the participants (EPINOLS/12-03-29/3proj6; OBESPAD/14-07-31/7proj3). Investigators did not participate in the statistical analyses and those responsible for the acquisition of different data were blinded to each other until discussion of the manuscript. Data were collected from selected patients referred to the surgery department of our hospital for the assessment of morbid obesity [body mass index (BMI) ≥ 40], who fulfilled the indications for the performance of laparoscopic sleeve gastrectomy in this period of time^[11]. Liver viral diseases were ruled out *via* standard laboratory analyses. Selected patients were clinically assessed immediately (< 24 h) before surgery and after a 12-mo period. Results for the standard techniques used in this study are illustrated in Figure 1.

Liver biopsy

We strictly followed the designated guidelines and recommendations set by the American Association for the Study of Liver Diseases^[12]. Portions of the liver were obtained during the surgical procedure to limit risks and the variability based on location of biopsy as well as to be certain of the accurate location of extraction. The procedure was performed with needle devices rather than wedge resection to avoid overestimates of fibrosis due to its proximity to the capsule. Liver biopsies after surgery (12 mo) were undertaken with a real-time image-guided percutaneous method immediately after MRI assessment. The above-mentioned guidelines include the accepted pathological features of fatty liver disease, especially the indices of inflammation (steatohepatitis) and fibrosis. Trained pathologists provided the relevant data using current histological scoring systems. Due to the low number of included patients and the need for parametric comparisons, we used percentage of fat for further analyses as previously described^[1]. Patients were considered free of steatosis when percentage of fat was $\leq 5\%$ ^[1] as evaluated using image analysis software (AnalySIS, Soft Imaging System, Munster, Germany)^[4].

MRI and MRS

MR data were acquired on a 1.5T Signa HDx MRI Scanner (General Electric Healthcare, Waukesha, WI) and included pre-surgical information regarding vascular structures. Patients fasted overnight and studies were performed in supine position using a whole-body radiofrequency coil for signal excitation

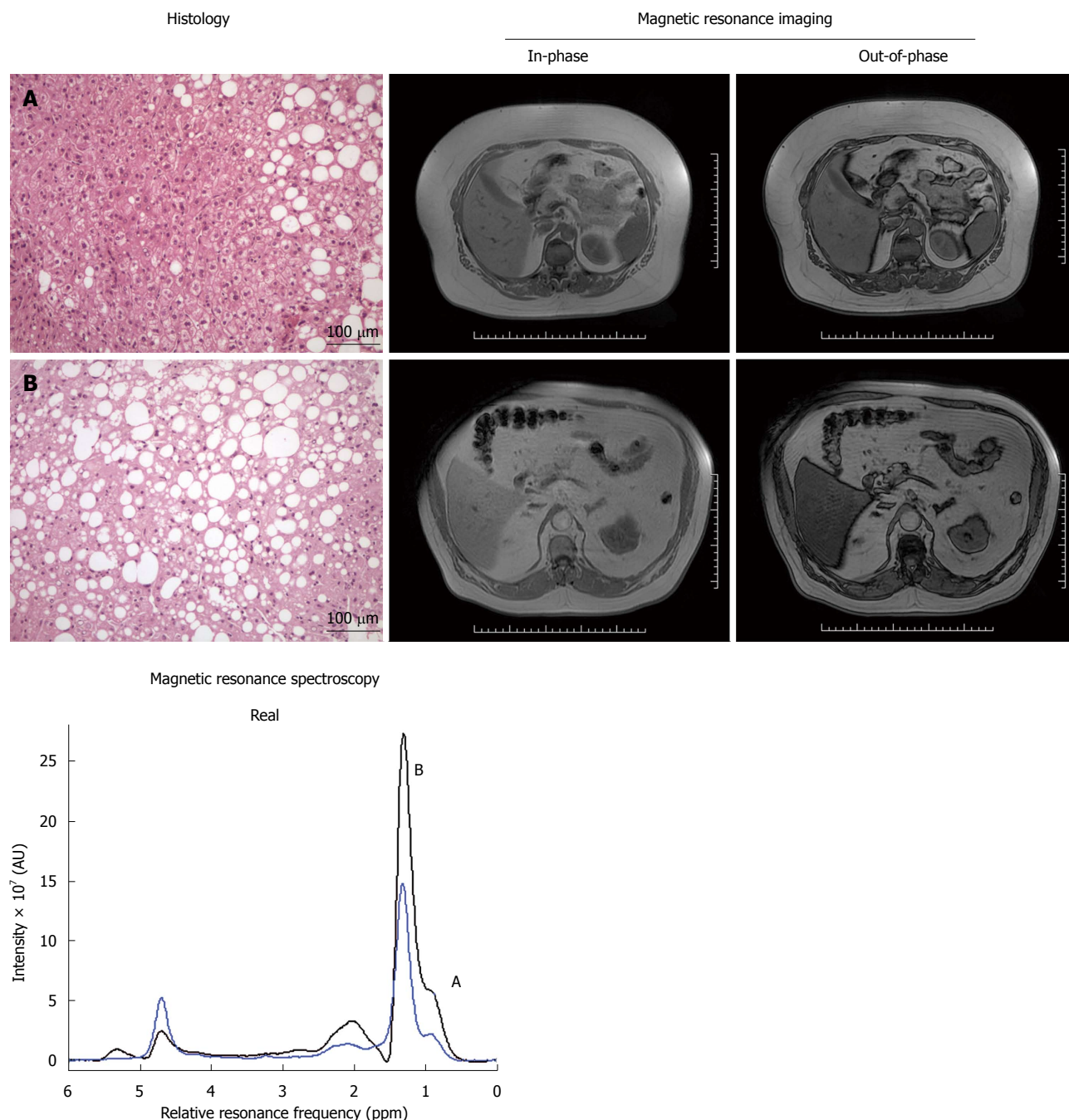


Figure 1 Representative results obtained with the methods described in patients with low (A) and severe fatty liver disease (B). The resulting spectra were drawn in blue and black, respectively. Scale bar in pictures under Histology title represents 100 μm (magnification $\times 100$).

and a 16-channel torso phased array coil for reception (General Electric Coils, Cleveland, OH). Imaging protocols^[13] consisted of: (1) coronal view (slice thickness 6 mm; gap 1 mm; flip angle 75°; matrix size 256 \times 192; field of view 48 cm); (2) axial view (slice thickness 7 mm; gap 1 mm; flip angle 75°; matrix 256 \times 192; field of view 48 cm); (3) axial T2 fast recovery Spin-Echo sequence with fat suppression (slice thickness 8 mm; gap 1 mm; matrix size 320 \times 224; field of view 48 cm); (4) axial T1-weighted dual phase gradient-echo sequence (repetition time in ms: 120/2.1 in opposed phase and 120/4.2 when

in phase; flip angle 80°; slice thickness 7 mm; gap 1 mm; matrix 256 \times 224; field of view 48 cm); and (5) a single-voxel breath-hold spectroscopy sequence. The Point-RESolved Spectroscopy^[14] sequence was used to record spin echos: acquisition with TR = 1500 ms, TE = 35 ms and 128 signal averages per data-frame with an eight-phase cycling scheme (2048 data points per spectroscopy frame with a spectral band width of 2500 Hz) resulting in an acquisition time of approximately 12 min. Chemical shift-selective radio frequency pulses ($n = 3$) suppressed the water signal. To discriminate the peaks corresponding to metabolites from artefacts,

each spectrum represents the average of 16 data-frame acquisitions (2 without water suppression)^[15]. To ensure the accurate biopsy site, the voxel was placed manually to provide accessibility and to avoid motion artefacts by the heart and flow artefacts from vascular structures^[13,16]. The biopsy procedure was carefully supervised according to this information. Values for signal intensity (SI) were obtained from pre-designed regions of interest (ROI) in the liver, spleen (both 20 mm × 20 mm) and paravertebral musculature (5 mm × 5 mm) and calculations were performed to avoid the possible T2 effect caused by ferric deposits. The hepatic fat fraction was measured according to the formula: $SI_{in-phase} - SI_{out-of-phase} / 2SI_{in-phase}$ ^[14,16-18]. The visceral and subcutaneous adipose tissues were quantified in out-of-phase images at pre-designed ROI. The MRS spectra were transformed using specific software via the SAGE (Spectroscopy Analysis General Electric) single voxel PROBE-Q reconstruction and normalized by water signal. The amplitude and area under the curve of each detectable peak were calculated as described^[19].

¹H NMR based metabolomics assays and laboratory procedures

Portions of hepatic tissue obtained from liver biopsy were flash-frozen and homogenized^[20]. For NMR measurements hydrophilic and lipophilic metabolites were separately lyophilized overnight to remove water and reconstituted as described^[10,21]. One- and two-dimensional ¹H NMR spectra were obtained at a 600.20 MHz frequency using an Avance III-600 Bruker spectrometer equipped with an inverse TCI 5 mm cryoprobe (Bruker, Rheinstetten, Germany). The frequency spectra were baseline corrected, and then calibrated using TopSpin software (version 2.1, Bruker). Resonance assignments were performed as described^[10,22]. Selected peaks were integrated using the AMIX 3.8 software package (Bruker) and the absolute concentration of single metabolites measured as described^[23] (Table 1). The integral at 0.87 corresponding to ω -CH₃ was used as a reference for total fatty acid chains to estimate the molar percentage of fatty acid signals. Common laboratory measurements were performed with commercially available reagents and well-established methods^[24,25]. The liver-tissue content of cholesterol and triglyceride was also determined after lipid extraction with the isopropyl alcohol-hexane method^[26]. Cytokines and adipokines were measured using enzyme-linked immunosorbent assays following the manufacturer's instructions (RD Systems, Minneapolis, United States).

Statistical analysis

Unless otherwise stated, quantitative variables were expressed as mean and interquartile range and categorical variables as absolute or relative frequencies. Statistical significance was considered if *P* values

were less than 0.05 and all analyses were conducted using the Statistical Package for Social Sciences (SPSS-18.0, Chicago, IL). Differences between groups were assessed with a Student's *t*-test, Kruskal-Wallis or Mann Whitney *U*-test. Spearman or Pearson correlation coefficients were used to evaluate the degree of association between variables. To assess differences before and after surgery, calculations were performed with Welch's *t*-tests and/or Wilcoxon's rank sum tests as well as ANOVA for repeated measures. To correct for multiple testing we used the False Discovery Rate estimated using the *Q*-value. The choice of tests was based on normality of values and the sample size, which varies with losses in observations as indicated in results.

RESULTS

Baseline and follow-up characteristics of participants

No acute illness or surgical complications were recorded during the study. Most patients were female (79%), with ages between 34 and 55 years. The reference BMI was calculated immediately before surgery (range: 42.1-51.2 kg/m²) and changes were negligible in the previous 6 mo. The body weight decreased in all patients. As a group, the mean decrease was 41.2 kg, approximately 35%. There were changes in parallel with weight loss, especially improvements in glucose and lipid metabolism, and in the circulating levels of leptin and adiponectin and the paraoxonase activity. Some inflammatory cytokines also decreased with weight loss, including C-reactive protein, but others did not. Contrarily, changes in the activities of enzymes traditionally considered as surrogates of liver function improved after surgery but independently of weight loss (Table 2, *n* = 19).

Bariatric surgery and remission of NAFLD

Despite morbid obesity, NAFLD was not a universal finding and severity was modest as assessed histologically (< 15% of triglyceride accumulation in 30% of the patients). In some it was negligible (< 5%) and in others severe (> 70%). This distribution was not explained by metabolic derangements. We discarded the presence of NASH as we did not observe hepatocyte ballooning, lobular inflammation or significant fibrosis. The correlation between histological percentages of triglyceride accumulation and hepatic fat fraction obtained by MRI was considered excellent (Figure 2A). One year after surgery, NAFLD decreased in all patients and was virtually absent (0%-7%) (Figure 2B; Table 2). In this second observation, with reduced values, the association between methods was also excellent. Sensitivity and specificity to stage the disease were sufficient (both > 90%). These data are probably the result of the continuous cooperation between radiologists and surgeons and the correction for T2 shortening due to liver iron deposition. The

Table 1 Absolute concentration of single metabolites obtained by ¹H NMR metabolomic profiles

	Mean	SD	Kruskal-Wallis (P)	None		Low		Moderate		Severe		U Mann-Withney (P)				
				Mean	SD	Mean	SD	Mean	SD	Mean	SD	N vs L	L vs M	M vs S	L vs S	
Lipid-soluble metabolites (mmol/g liver)																
Total cholesterol	5.19	1.71	0.008	2.74	0.51	5.03	2.15	5.89	0.68	5.99	1.06					
Free cholesterol	1.32	0.40		1.09	0.18	1.52	0.41	1.36	0.42	1.00	0.27					
Sterified cholesterol	3.87	1.65		1.66	0.70	3.50	1.82	4.54	1.01	4.99	1.31					
Triglycerides	141.33	92.95		13.71	6.33	88.82	59.32	184.61	26.11	259.29	67.09		0.017			0.020
Diglycerides	8.04	3.22		10.35	7.42	8.40	2.37	8.55	2.70	4.91	0.96					0.039
Monoglycerides	0.73	0.20	0.69	0.25	0.80	0.28	0.77	0.12	0.57	0.06						
Total phospholipids	22.75	5.86	0.046	29.44	7.74	24.28	1.56	23.75	1.65	13.59	6.03			0.025		0.020
PC	15.90	4.06		20.05	5.70	16.71	1.33	17.13	0.73	9.50	4.21			0.025		0.020
PE	10.59	2.31		12.37	2.58	11.13	0.33	11.52	0.79	6.79	2.75			0.025		0.020
Phosphatidylinositol	1.99	0.85	0.028	1.54	0.65	2.76	0.71	1.77	0.60	1.13	0.22		0.028			0.020
Phosphatidylserine	0.13	0.07		0.07	0.01	0.14	0.08	0.15	0.07	0.10	0.03					
Lysophosphatidylcholine	1.07	0.75		0.62	0.07	0.82	0.20	1.59	1.22	1.02	0.25		0.045			
Sphingomyelin	1.97	0.39		2.18	0.53	2.13	0.22	2.03	0.24	1.41	0.37					0.020
Plasmalogen	1.01	0.13		0.97	0.23	1.01	0.06	1.10	0.08	0.88	0.17					
Lipid-soluble metabolites (%mol)																
Omega-3 fatty acids	2.14	1.32	0.009	4.57	0.40	2.49	1.15	1.44	0.10	0.98	0.32		0.028			0.020
Oleic acid (18:1 ω-9)	24.53	4.37		16.42	1.70	24.34	4.29	26.62	1.15	26.81	2.95	0.045				
Arachidonic (20:4 ω-6)	3.51	3.09	0.009	9.82	2.89	3.96	2.16	1.94	0.32	1.05	0.36		0.045	0.025		0.020
+ eicosapentaenoic (20:5 ω-3) acids																
Docosahexaenoic acid (22:6 ω-3)	0.72	0.65	0.012	1.97	0.41	0.85	0.56	0.39	0.09	0.20	0.14		0.028			0.027
Linoleic acid (18:2 ω-6)	5.91	1.81	0.02	9.63	0.23	6.23	0.84	5.02	0.98	4.27	0.85	0.045				0.020
PUFA	20.41	17.58	0.009	55.73	12.60	23.43	12.9	11.55	1.71	5.57	3.11		0.028			0.020
MUFA, with PUFA	93.15	11.47	0.008	112.96	3.53	97.79	5.27	87.68	5.94	79.81	6.57	0.045	0.028			0.020
PUFA/MUFA ratio	0.20	0.14	0.015	0.49	0.09	0.23	0.11	0.13	0.02	0.07	0.03					0.020
PC/PE ratio	1.48	0.09		1.61	0.12	1.50	0.08	1.48	0.06	1.38	0.06					
Aqueous-soluble metabolites (mmol/g liver)																
Glucose-6-phosphate	32.65	13.3		41.69	32.96	35.63	7.94	34.27	4.46	17.96	12.1					
Glycogen	2.19	2.34		2.57	0.62	1.70	0.81	2.95	4.11	1.66	1.67					
UDPG	1.22	0.50		1.74	0.75	1.25	0.14	1.19	0.54	0.89	0.73					
3-pyruvate	1.72	0.68		2.24	1.21	1.93	0.49	1.75	0.45	0.92	0.63					
3-hidroxybutyrate	0.76	0.35		1.04	0.86	0.71	0.25	0.69	0.25	0.78	0.40					
Lactate	20.48	6.63		17.37	5.60	23.75	3.23	20.95	6.56	15.25	10.9					
Fumarate	0.30	0.10		0.32	0.06	0.36	0.12	0.28	0.06	0.21	0.05					0.038
Free glycerol	1.64	0.94		1.53	0.28	1.48	0.26	1.47	0.39	2.35	2.29					
NAD/NADH/NADPH	0.39	0.13		0.50	0.18	0.46	0.07	0.35	0.10	0.26	0.11					0.038
ATP/ADP/AMP	1.68	0.70		2.43	1.30	1.86	0.46	1.55	0.59	1.02	0.52					0.038
UTP/UDP/UMP	0.87	0.29		0.85	0.21	1.03	0.23	0.97	0.20	0.43	0.11			0.025		0.020
Uracil	0.04	0.02		0.03	0.01	0.05	0.02	0.04	0.01	0.02	0.02					
Carnitine	1.16	0.59		2.16	0.82	1.11	0.35	1.02	0.50	0.83	0.56					
Cholines	1.50	0.36		1.62	0.05	1.59	0.27	1.58	0.31	1.07	0.50					
Acetates	0.48	0.11		0.45	0.02	0.54	0.08	0.49	0.13	0.39	0.12					
Ascorbic acid	1.36	0.57		1.80	0.40	1.30	0.38	1.36	0.62	1.18	1.01					
Leucine	0.55	0.10		0.50	0.02	0.59	0.09	0.59	0.10	0.44	0.02			0.025		0.020
Valine	0.60	0.13		0.55	0.01	0.66	0.09	0.65	0.12	0.42	0.07			0.025		0.020
Alanine	6.07	2.38		5.68	4.18	7.81	1.32	5.83	0.83	3.22	2.54		0.028			0.038
Isoleucine	0.25	0.05		0.23	0.01	0.26	0.04	0.29	0.06	0.19	0.02			0.025		
Lysine	1.26	0.26		1.21	0.08	1.38	0.13	1.34	0.33	0.92	0.14					0.020
Glutamine	3.96	1.59		5.83	1.90	4.48	0.89	3.60	1.19	2.27	1.86					
Glutamate	4.42	1.63	0.038	4.70	0.01	5.63	1.78	3.92	0.82	2.65	0.98					0.020
Methionine	0.80	0.24		0.76	0.24	0.84	0.23	0.70	0.18	0.90	0.42					
Phenylalanine	0.26	0.05		0.25	0.02	0.29	0.03	0.27	0.04	0.17	0.05					0.020
Threonine	1.46	0.50		2.13	0.59	1.58	0.45	1.36	0.08	0.94	0.53					
Tyrosine	0.20	0.05	0.026	0.16	0.04	0.24	0.02	0.21	0.04	0.14	0.05	0.045				0.020
Histidine	0.16	0.07		0.25	0.13	0.17	0.06	0.13	0.04	0.12	0.04					
Taurine	12.53	4.07		17.03	1.55	13.68	4.14	11.57	1.46	8.81	5.38					
Gluthathione oxidized	0.73	0.40		1.31	0.46	0.63	0.29	0.57	0.38	0.78	0.42					
Creatine	3.16	1.24		4.39	0.78	3.74	0.73	2.89	0.91	1.61	1.37					0.038
Creatinine	0.15	0.04	0.010	0.15	0.01	0.19	0.02	0.12	0.02	0.10	0.05	0.045	0.006			0.020

PC: Phosphatidylcholine; PE: Phosphatidylethanolamine; PUFA: Polyunsaturated fatty acids; UDPG: Uridine diphosphate glucose; MUFA: Monounsaturated fatty acids.

Table 2 Selected variables to explore metabolic changes and anti-oxidative or anti-inflammatory effects prior to and one year after surgery

	Before surgery	After surgery	P value
BMI, kg/m ²	45.4 (44.1-47.0)	32.3 (30.1-35.4)	< 0.001
Liver steatosis, %	30 (5-55)	6 (0-15)	< 0.001
Glucose, mmol/L	7.9 (6.7-10.1)	5.4 (4.6-6.2)	< 0.001
Insulin, pmol/L	114.2 (86.3-166.8)	66.9 (58.0-70.1)	< 0.001
HOMA2-IR	4.73 (1.85-10.67)	1.95 (1.30-5.80)	< 0.001
Total cholesterol, mmol/L	4.93 (4.59-5.24)	4.28 (4.05-4.87)	< 0.001
Triglycerides, mmol/L	1.76 (1.12-2.42)	1.08 (0.85-1.38)	< 0.001
HDL-cholesterol, mmol/L	1.06 (0.94-1.23)	1.31 (1.02-1.44)	< 0.001
NEFAs, mmol/L	1.08 (0.85-1.48)	0.80 (0.61-0.96)	< 0.050
Bilirubin, mmol/L	8.2 (5.1-9.1)	9.1 (7.2-8.5)	NS
ALP, μ Kat/L	0.82 (0.70-1.1)	0.65 (0.54-0.92)	< 0.050
AST, μ Kat/L	1.03 (0.37-1.24)	0.82 (0.24-1.14)	NS
ALT, μ Kat/L	0.52 (0.43-1.25)	0.45 (0.33-0.97)	NS
GGT, μ Kat/L	0.34 (0.24-1.08)	0.30 (0.20-0.95)	NS
Amylase, μ Kat/L	0.67 (0.54-0.84)	0.42 (0.33-0.65)	< 0.050
Paraoxonase, U/L	214 (160-410)	360 (200-405)	< 0.050
Leptin, ng/mL	32.5 (21.2-40.1)	9.2 (6.3-11.1)	< 0.001
Adiponectin, ng/mL	4.2 (2.1-5.9)	7.5 (4.9-8.2)	< 0.001
Interleukin 6, pg/mL	0.52 (0.21-0.75)	0.43 (0.12-0.59)	NS
Interleukin 8, pg/mL	1.86 (1.45-2.15)	1.42 (1.22-1.65)	NS
TNF- α , pg/mL	6.21 (4.35-7.82)	5.12 (3.95-7.36)	< 0.050
CCL2, pg/mL	510 (425-590)	395 (280-430)	< 0.001
Hs-CRP, mg/L	4.6 (1.5-7.2)	1.1 (0.3-1.67)	< 0.001

We only included participants with a complete dataset ($n = 19$). Values are expressed as median (interquartile range); AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyl transpeptidase; HOMA2-IR: Homeostatic model assessment index; NEFAs: Non-esterified fatty acids; TNF- α : Tumor necrosis factor- α ; CCL2: Chemokine (C-C motif) ligand 2; Hs-CRP: High sensitivity C-reactive protein.

mathematical expression most likely will change among laboratories to ensure correct stage in severe cases. Additional corrections in spleen and muscles were apparently unnecessary (Figure 3). Further, the lack of sampling variability is paramount in comparisons because the heterogeneous distribution of NAFLD is relatively common in these patients. This fact has been illustrated in Figure 4.

Results obtained by MRS were similar to those observed by MRI

Contrary to our hypothesis, MRS did not provide additional and relevant information in the staging of NAFLD. Moreover, in some patients without NAFLD, some peaks were undetected and inter-individual variability of other peaks was too high to be considered. However, there were at least 3 peaks with reliable information to compare values before and after surgery (the 0.9-ppm, 1.3-ppm peak and 2.2 ppm peak, Figure 5) most likely indicating the presence of -CH₃ (0.9 ppm), -CH₂- (1.3 ppm) and -CH=CH- (2.2 ppm) (Figure 6). The calculations of either amplitude or the area under the curve (AUC) yield similar results with fair-to-good correlation with histologically assessed values

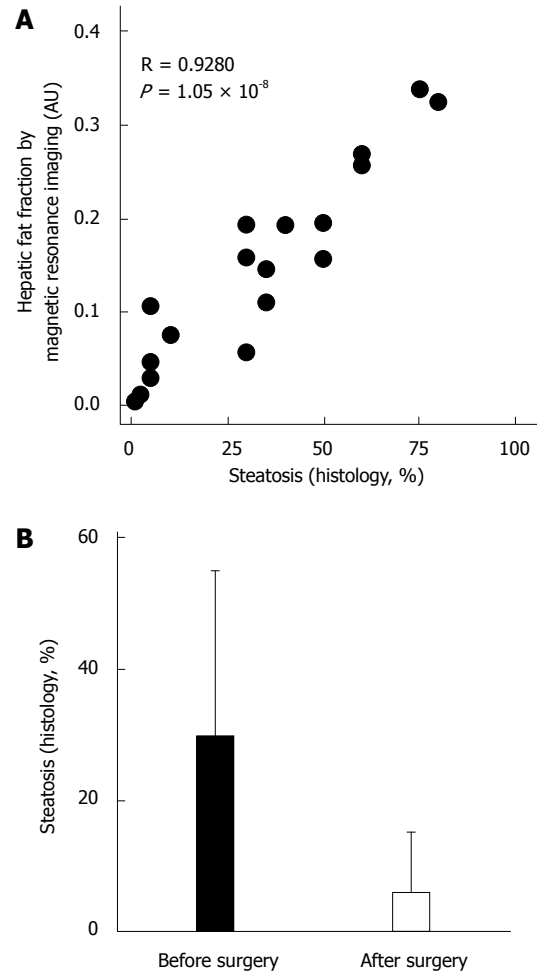


Figure 2 Correlation between hepatic steatosis measured by magnetic resonance imaging and by histological analysis before surgery (A); non-alcoholic fatty liver disease was considerably reduced after one year in all patients (B).

but less robust than those observed with MRI (Figure 7). Characteristically, the 1.3-ppm and 2.2-ppm peaks are considered as representative of triglyceride and unsaturated fatty acids, but actual values obtained by biochemical measurements (see below) suggest the combination of multiple signals from other compounds.

Metabolomics: Liver fat composition and the assessment of steatosis

The correlation between steatosis and liver triglyceride content was not different from that obtained between steatosis and MRI ($r = 0.89$, $P = 1.2 \times 10^{-5}$, $n = 16$) indicating that the invasive measurement of liver triglyceride content is not a valuable diagnostic tool in these patients. Data provided by ¹H NMR spectroscopic profiles are given only for the biopsy obtained during surgery because in some samples obtained in the second biopsy the concentrations of most metabolites were lower than a predesignated detection level. Hence, this portion of the liver was used only for clinical purposes. ¹H NMR spectra of aqueous soluble liver tissue extracts showed resonances associated

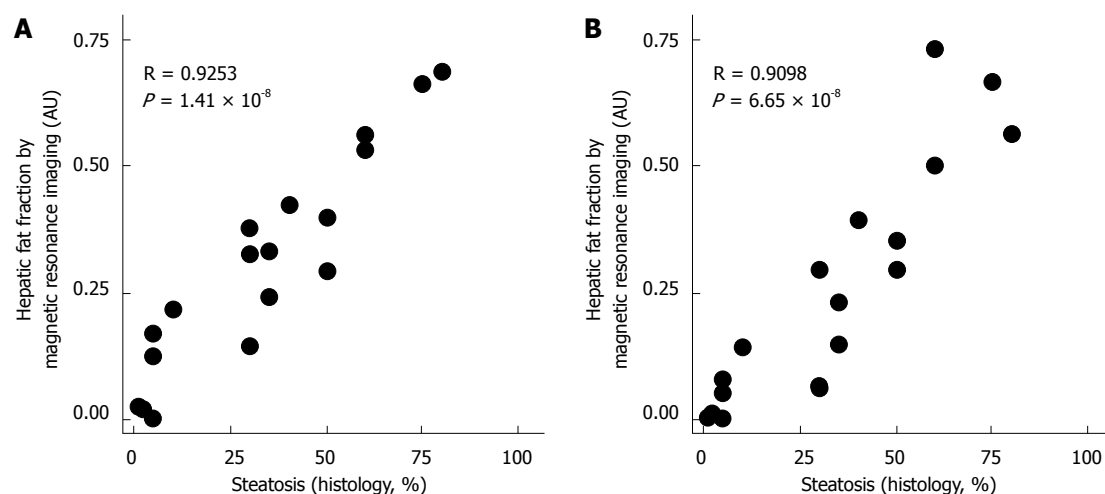


Figure 3 Magnetic resonance imaging signal was further corrected by the spleen (A) or muscle (B) intensity signals, but this did not improve the quality of the correlation with hepatic steatosis assessed by histological analysis.

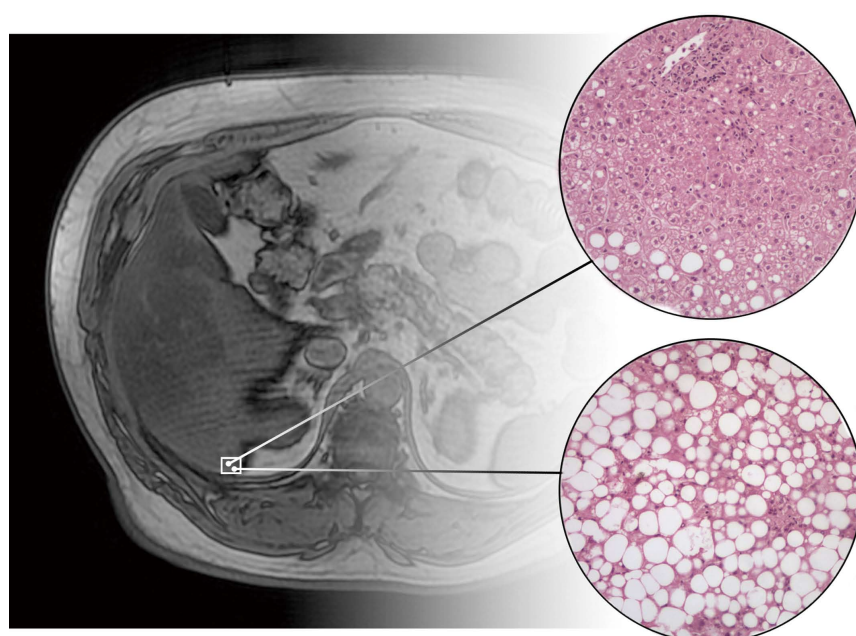
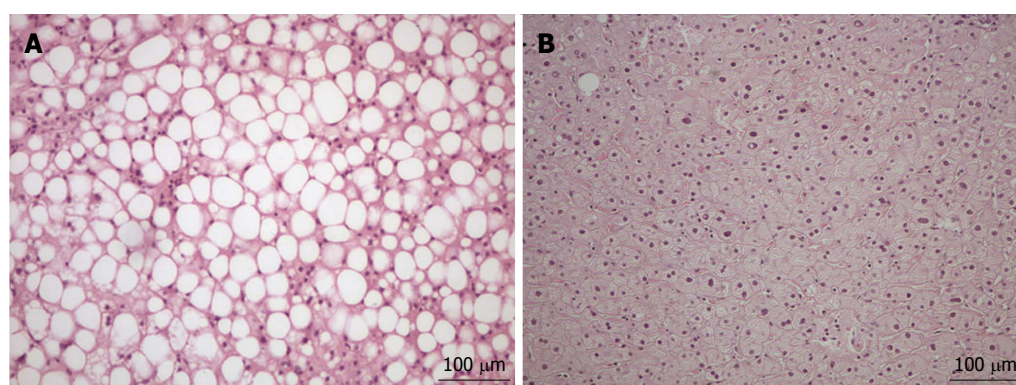


Figure 4 Liver fat distribution is commonly non-homogeneous in non-alcoholic fat liver disease, and this fact could influence the diagnosis of steatosis.



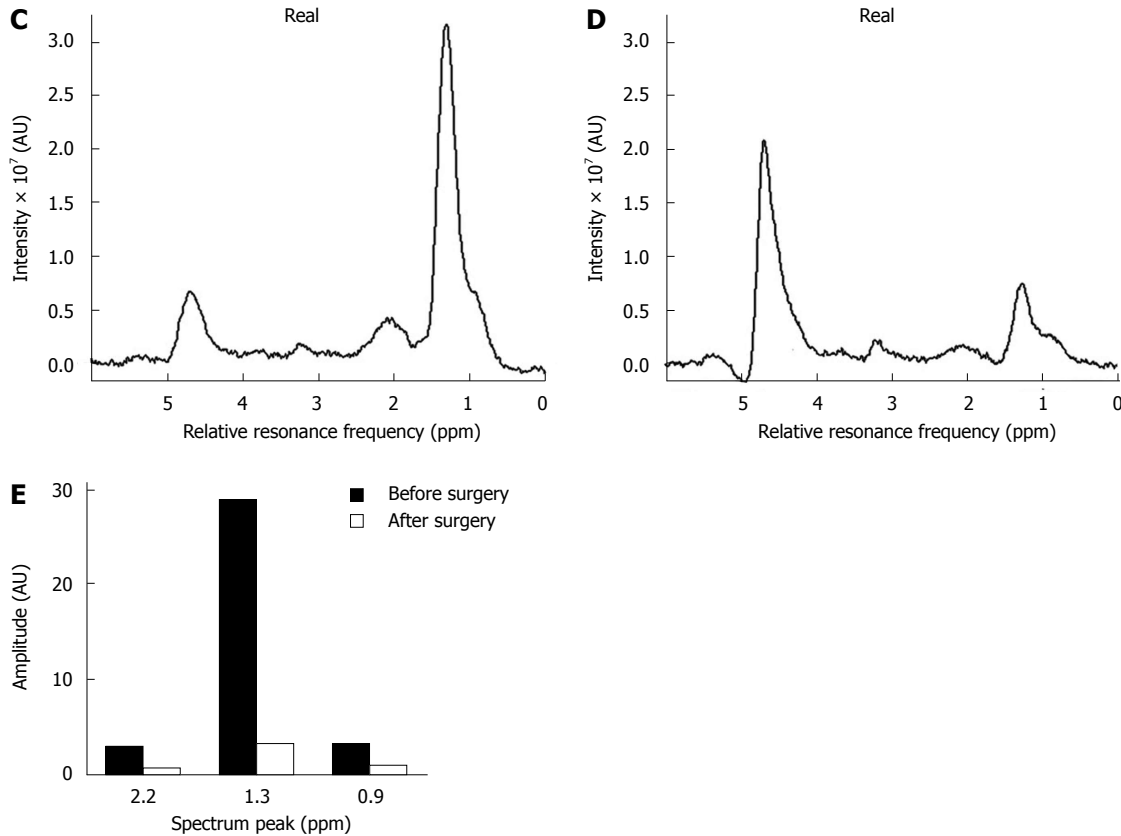


Figure 5 Hepatic steatosis was evaluated in patients before and after surgery in liver biopsy (A, B); the decrease in hepatic fat content also could be visualized by magnetic resonance spectroscopy (C, D), where the peaks that offer more information were those at 0.9, 1.3 and 2.2 ppm (E). Scale bar in A and B represents 100 μ m (magnification \times 100).

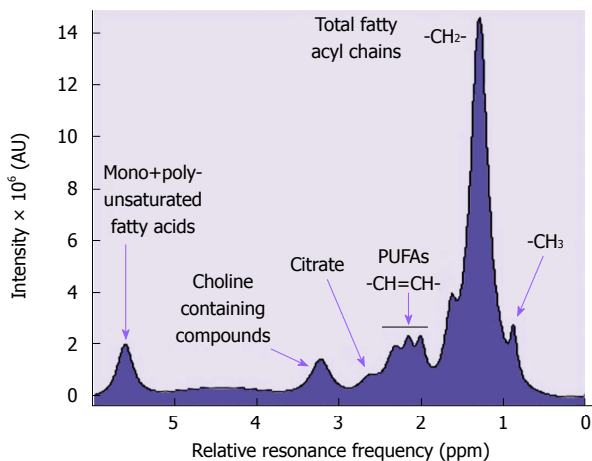
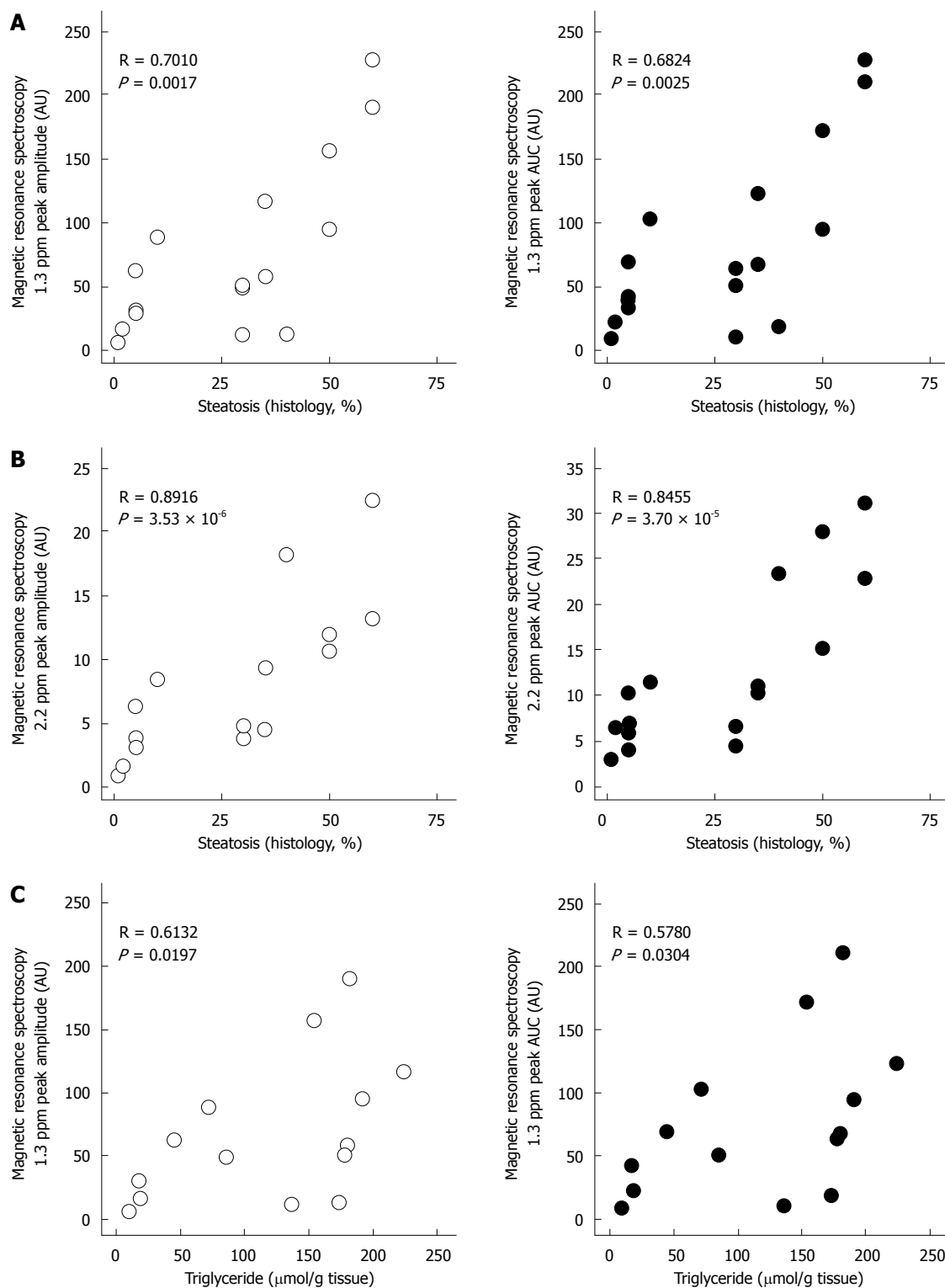


Figure 6 Representative spectrum of identifiable metabolites with magnetic resonance spectroscopy *in vivo*: 0.9 ppm - terminal carbon ($-\text{CH}_3$); 1.3 ppm - total fatty acyl chains ($-\text{CH}_2-$); 2.0-2.2 ppm - polyunsaturated fatty acids ($-\text{CH}_2=\text{CH}_2-$); 2.6 ppm - Citrate; 3.2 ppm - choline containing compounds; 5.3-5.6 ppm - mono+polyunsaturated fatty acids. Water relative resonance frequency appears at 4.7 ppm as major compound. In this case, water is suppressed for facilitate the visualization of other detectable compounds.

with low molecular weight metabolites, and ^1H NMR spectra from lipid soluble extracts depicted several dominating regions with major peaks attributable

to double bonds mainly from protons belonging to di- or triacylated glycerols, the phospholipids polar head groups, methylene, methyl groups of the fatty acyl chains and unsaturated fatty acyl moieties, and they were all associated with liver fat deposition (Table 1). Interestingly, in patients with no or very moderate NAFLD, the levels of triglycerides and oleic acid were low and there were increased hepatic levels of polyunsaturated fatty acids (PUFA) ω -3 fatty acyls, docosahexanoic 22:6 (n-3) (DHA), arachidonic 20:4 (n-6) (ARA) + eicosapentaenoic 20:5 (n-3) (EPA), and the ratio between poly- and mono-unsaturated fatty acids (PUFA/MUFA). These changes paralleled the increased levels of glycerol, lactate, glucose-6-phosphate (G-6-P), carnitine and taurine. Conversely, in moderate to severe steatosis, the levels of triglyceride, oleic acid, and some amino acids were high but there was a significant depletion of PUFA. This is illustrated by significantly positive or inverse correlations found in these metabolites with the stage of NAFLD (Figure 8). Importantly, in severe NAFLD there were significant reductions in diglycerides (DAG), G-6-P, fumarate, NAD/NADH, ATP/AMP, and UTP/UMP (Table 1) most likely indicating a dysfunction in the coordination of mitochondrial fat oxidation and glucose transport activity.



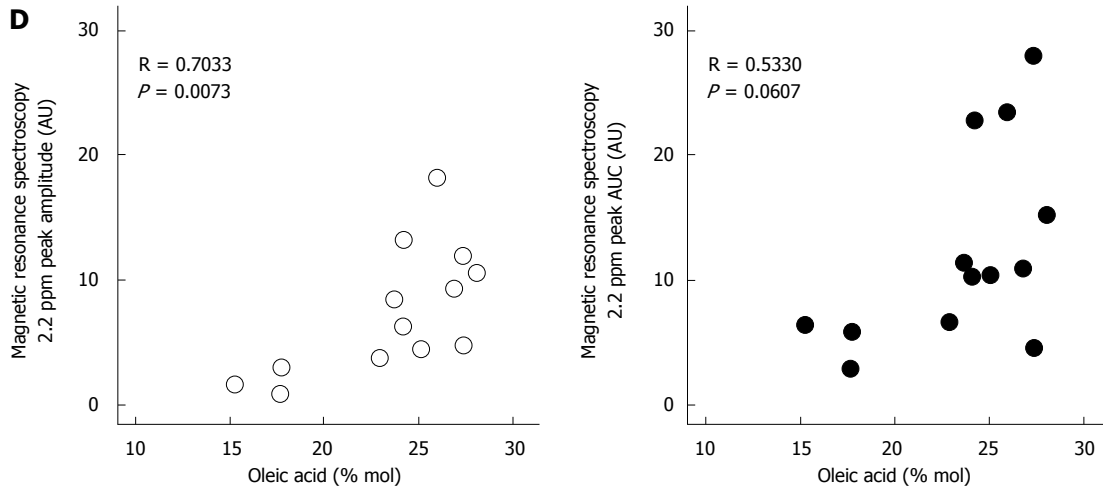
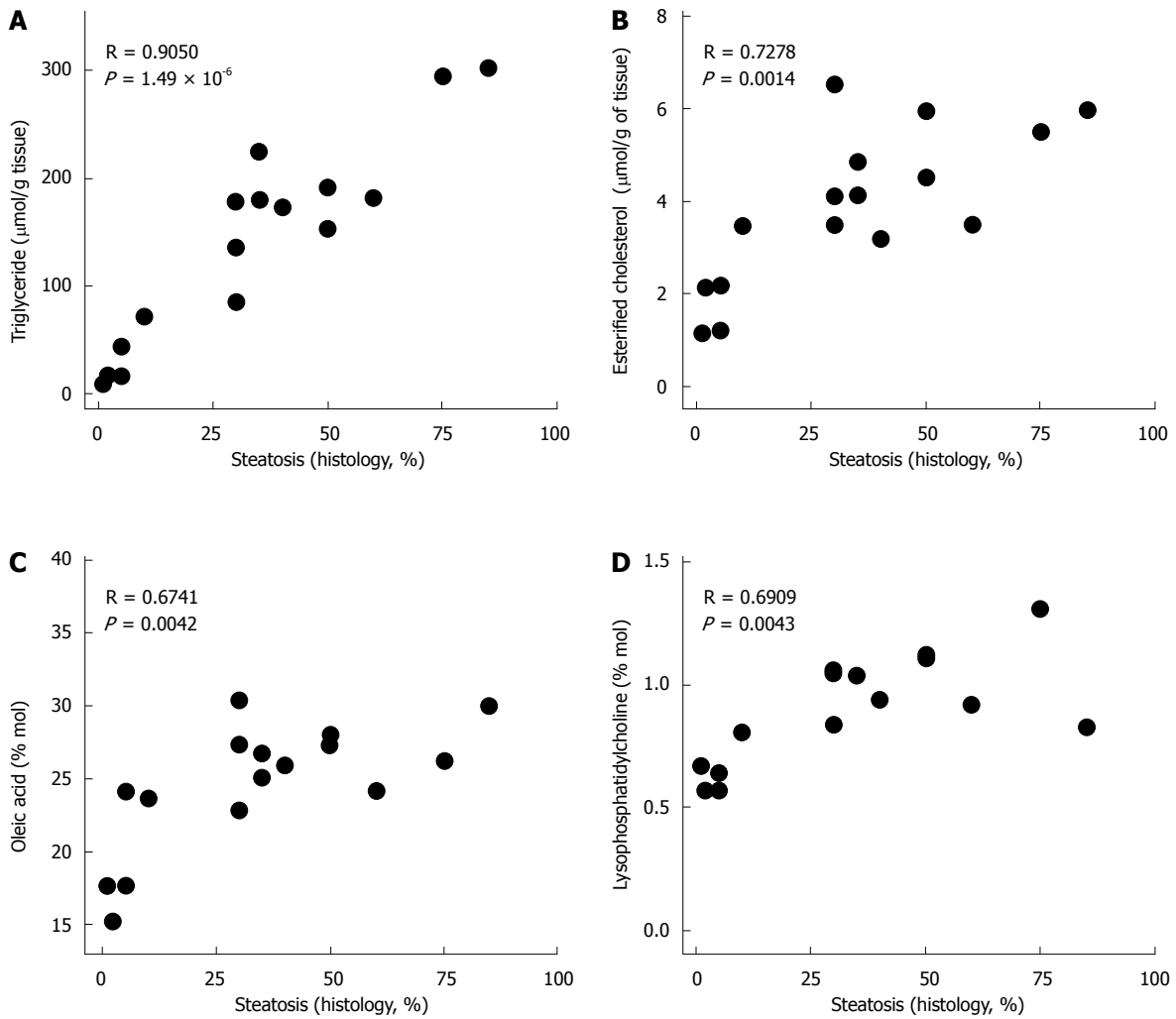


Figure 7 Similar results were obtained using the following methods of calculation: Amplitude of the peaks measurement (left open-circle plots) and area under the curve (AUC, right black-circle plots). The most important peaks associated with fat are at 1.3 ppm (A) and 2.2 ppm (B). 1.3 ppm peak is mainly associated with triglycerides (C) while 2.2 ppm peak is associated with unsaturations (D).



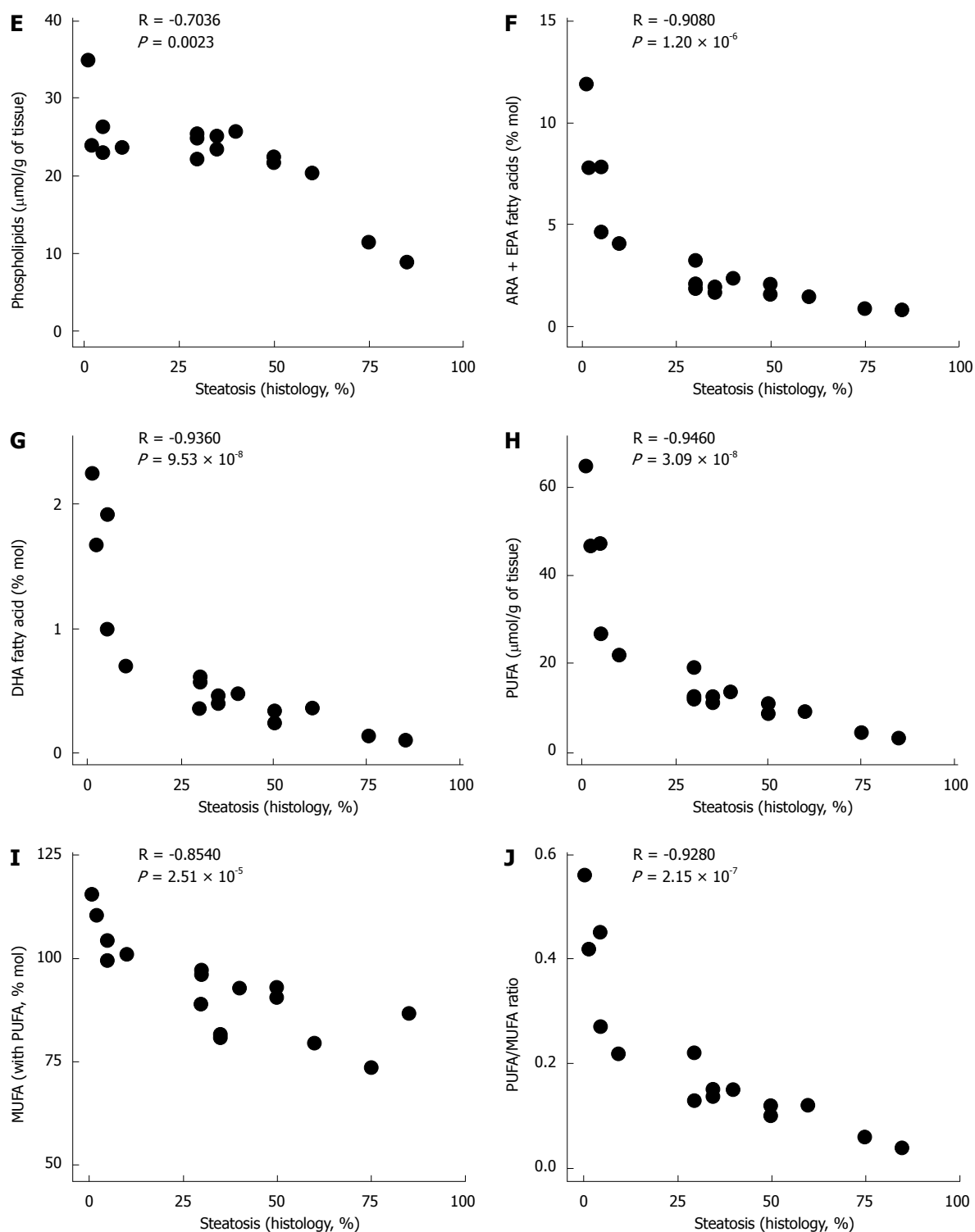


Figure 8 Several lipidic metabolites measured using ^1H NMR-based metabolomics were clearly positively associated with hepatic steatosis (A-D); Others showed the opposite behavior (E-J). ARA: Arachidonic acid; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid; PUFA: Poly-unsaturated fatty acids; MUFA: Mono-unsaturated fatty acids.

Dissociation between excess lipid storage in adipose tissue, NAFLD and insulin resistance

Our data indicate that even in patients with morbid obesity the incidence of NAFLD is not universal. Bariatric surgery was accompanied by a reduction in BMI and practically eliminated the ectopic fat in the liver but patients remained obese (BMI > 30).

There was no correlation between BMI and steatosis ($r = 0.227$). Additionally, body fat distribution was not associated with the development of fatty liver as the correlations between the subcutaneous and visceral adipose tissues, measured by MRI, and fatty liver severity were not different from 0 (Figure 9). Moreover, the degree of impaired glucose metabolism

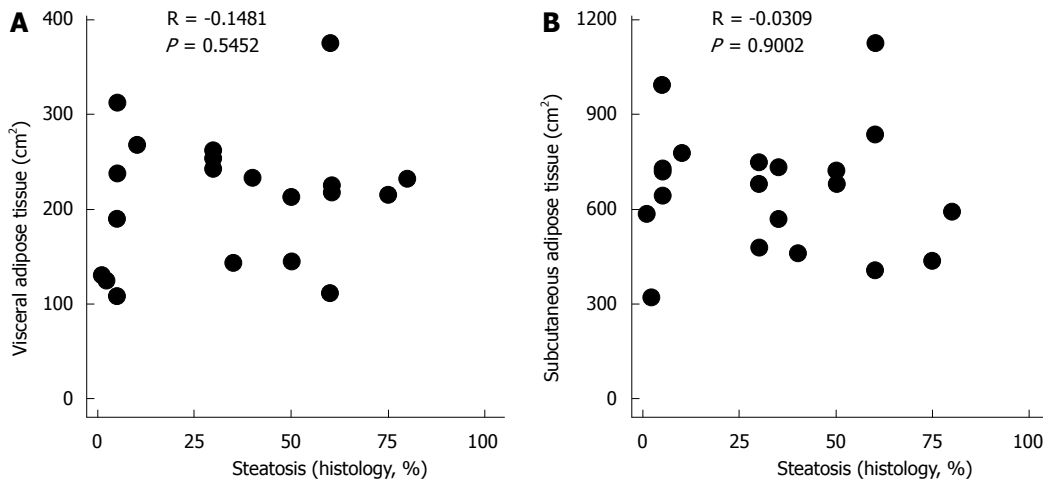


Figure 9 Body fat content was measured using magnetic resonance imaging. Both visceral (A) and subcutaneous adipose tissue (B) were not related to the development of non-alcoholic fatty liver disease.

of patients was not associated with the presence of NAFLD. There were insulin-sensitive individuals with a high percentage of ectopic liver fat and there were participants with insulin resistance remaining free of NAFLD. This is counterintuitive because the overflow of energy storage from adipose tissue to the liver is apparently unavoidable. Our findings reinforce the concept that the composition of ectopic lipid is important in searching for metabolites most likely mediating defects in insulin signalling and/or mitochondrial function^[6,10,27-32].

DISCUSSION

The metabolic syndrome and obesity are strong predictors of NAFLD but mechanisms to explain an association between NAFLD and morbid obesity remain unclear. Some, most likely 20%-30%, of obese patients are currently identified as "metabolically healthy"^[33] but this is irrelevant because it is not translated into a lower risk of mortality^[34]. NAFLD is not a benign condition and consequently should be carefully assessed. Our MRI data indicate that in patients with morbid obesity this technique provides diagnostic accuracy > 90% with sufficient sensitivity and specificity (both > 90%) to stage the disease and to assess the effect of surgery. The main limitations include the cost, the requirement for considerable efforts to minimize sampling variability and the size of the patients undergoing bariatric surgery. The latter is also the cause of limitations in our study, which should probably be extended to a more protracted time of observation.

In this group of patients, our data indicate that MRS, liver biopsy and the measurement of liver fat content do not provide clinically useful information and were considered either superfluous or redundant because the likelihood to find NASH was low. Further research, however, should include technical

improvements in the design of the apparatus and in the ability to obtain more detailed spectra. The advantage of this non-invasive technique is obvious but our study design precludes the possibility to test its value in differentiating NAFLD from NASH and/or significant fibrosis, which is important to assess prognosis and indicate management^[2,3,35]. However, we consistently observe superior effects on weight loss, NAFLD and lipid and glucose metabolic derangements using bariatric surgery as compared with intensive but conservative medical therapy. Further, bariatric surgery improves insulin sensitivity, lipid metabolism, inflammation, oxidative stress and most likely other factors that might contribute to NAFLD. Previous data suggest the speculation that rapid and sustained weight loss may also improve the histologic characteristics of NASH^[6,36,37] but even modest weight reduction (7%-10%) results in significant improvement of NASH^[38,39]. In addition, we provide further evidence that inflammatory factors, oxidative stress, and the liberation of cytokines from adipose tissue are likely modulators^[40].

Our data limit further the clinical indication of liver biopsy in obese patients for those with the additional presence of known factors affecting the progression of liver disease such as alcohol or drugs consumption, toxin exposure or viral disease. However, they also indicate that liver biopsy is critical to obtain research material to uncover the precise molecular mechanisms that remain elusive. Particularly, we observed a significant mobilization in metabolites according to the severity of NAFLD. We used ¹H NMR spectra to obtain signals with technology similar to both MRI and MRS^[10] but future studies should include analytical methods that increase the number of reliably detected metabolites^[4,22]. This is important because research may result in alternative therapeutic strategies. Of note, we have found that severe NAFLD is accompanied by the progressive inhibition of fatty acid oxidation, which

increases glucose oxidation and decreases glucose formation in fatty liver, indicating both mitochondrial dysfunction and the existence of metabolites that cause defects in insulin signalling or glucose transport activity^[4,31,41]. The consideration of NAFLD as a likely mitochondrial disease^[42,43] is important and confirms our previous findings in animal models characterized by the accumulation of damaged mitochondria and mitochondrial fragmentation^[30,44], apparently involved in response to liver inflammation, excess of energy intake and autophagy^[45,46]. The implications of a change in paradigm might be also of clinical value. Obesity down-regulates liver autophagy and the therapeutic target should be the restoration of liver autophagy. This is also important due to the clear dissociation between lipid storage in adipose tissue, NAFLD and insulin resistance. This is in line with the Randle cycle, which draws attention to competition between glucose and fatty acids for their oxidation, and pharmacological models of mitochondrial impairment, which stimulates insulin sensitivity and protects against diet-induced obesity, NAFLD and insulin resistance^[47,48]. However, an important limitation of our study is the relatively low amount of valid data and we cannot exclude the possibility of other important metabolic factors. It may also be useful to focus the search for serum biomarkers among metabolites involved in energy metabolism^[4,49]. It is likely that a unique technique may not suffice and serum biomarkers should be combined with MRI or other techniques (e.g., elastography) to provide the optimal diagnostic discrimination that is required in the fight against obesity-associated disorders^[15]. Interestingly, these data are partially confirmative of findings indicating that metabolic changes are weight-dependent. However, in the assessment of NAFLD progression these may be limited to the morbidly obese patient^[50].

To sum up, MRI is a sufficient, non-invasive technique, adequate to accurately stage NAFLD in obese patients and to assess the clinical follow-up of bariatric surgery. In uncomplicated patients, when procedures are performed to avoid sampling variability and confounding factors, data from MRS, liver biopsy and the measurement of liver fat content may be considered superfluous. Data from metabolomics may be useful to explain the dissociation between excess lipid storage in adipose tissue, NAFLD and insulin resistance. Additionally, they may support the consideration of NAFLD as a likely mitochondrial disease, which is important to fully understand the pathogenesis and to focus the search for biomarkers and therapeutic strategies.

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the manuscript.

COMMENTS

Background

The prevalence of non-alcoholic fatty liver disease (NAFLD) is constantly increasing and is associated with an upsurge in overweight and obesity. Early detection is important because fat deposition in the liver is a recognized risk for cardiovascular health and may progress to severe liver disease. On the other hand, the reversal of this condition is beneficial.

Research frontiers

The metabolic mechanisms leading to NAFLD are not well understood. In obese patients, weight loss modifies its course considerably but to achieve this effect is currently a considerable clinical challenge. Under certain conditions bariatric surgery is an effective treatment to obtain a rapid and sustained weight loss. Unfortunately, liver biopsy remains the only available method to obtain useful information. The consequence is that early diagnosis of NAFLD is difficult. In this study, the authors explore the safety and specificity of MRI in providing accurate diagnosis.

Innovations and breakthroughs

The previous application of non-invasive procedures to ascertain some clinical aspects of NAFLD has been considered suboptimal. The authors devised imaging protocols taking into account all technical limitations and they found them useful especially in bariatric surgery, which requires pre-surgical information regarding vascular structures. These resulted in excellent correlations between histological percentages of triglyceride accumulation and hepatic fat fraction obtained by magnetic resonance imaging (MRI). Using some sophisticated spectroscopic analyses, they have concluded that beneficial effects in associated metabolic conditions achieved by bariatric surgery are apparently unrelated to the decrease in body weight. Some data also indicate the relevance of mitochondrial function in the pathogenesis of ectopic fat deposition and potentially new therapeutic perspectives.

Applications

MRI is an adequate non-invasive technique, to accurately stage fatty liver disease in the clinical follow-up of bariatric surgery. Associated technologies may be used in the assessment of other clinical conditions and to clarify the actual mechanisms of fatty liver disease.

Terminology

Fat should be stored in adipose tissue. The presence in the liver represents a serious metabolic disturbance. The authors have used nuclear magnetic resonance, a physical phenomenon in which nuclei in a magnetic field absorb and re-emit electromagnetic radiation, to obtain images and relevant data on the composition of tissues.

Peer-review

This is a good observational study in which the authors evaluated the efficacy of MRI in NAFLD. The results are interesting and suggest new metabolic insights. An important limitation is the relatively low number of patients included. Further research should be directed to confirm these findings and to generalize its clinical use in obesity-related conditions.

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